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12 a. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for public release; distribution unlimited.			12 b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)  The primary goal of this program was to identify and develop a novel compound with antibiotic activity against anthrax. We initially focused on the development of borinic acid esters. The early leads did not prove to have sufficient attributes to be developed as therapeutics. Efforts then focused on optimizing the compounds to possess better pharmacokinetics and lower protein binding. By May, 2004, compounds were synthesized with low protein binding, good bioavailability, long half lives and good tissue penetration. Unfortunately, in rodent anthrax models the best compound totally protected only 20% of the mice inoculated with a lethal anthrax dose. It was clear from these studies that a high volume of distribution was needed for <i>in vivo</i> efficacy. Based on this finding we focused instead on AN0900, a semi-synthetic glycopeptide antibiotic with a very large volume of distribution that worked by inhibiting transglycosylase. Initial studies in both the intravascular vegetative anthrax and the pulmonary anthrax mouse models demonstrated that AN0900 administration 1 h after anthrax inoculation provided complete protection using a single daily dose of 10-25 mg/kg. In conclusion, within the 2 years of the contract, AN0900 was discovered and represents a novel compound working by a unique mechanism of action.				
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**(Continuation Sheet)**

**(1) List of papers submitted or published under ARO sponsorship during this reporting period.**

None.

**(2) Scientific personnel supported by this project and honors/awards/degrees**

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**(3) Report of Inventions**

We have filed fifteen provisional applications to cover the new structures we synthesized and tested as antibiotics:

- 1) US Provisional Application No. 60/434,375 (December 18, 2002) "Borinic Acid Antibiotics"
- 2) US Provisional Application No. 60/436,095 (December 23, 2002) "Azole Borinate Antibiotics"
- 3) US Provisional Application No. 60/436,096 (December 23, 2002) "Amino Acid-Borinic Acid Antibiotics"
- 4) US Provisional Application No. 60/437,849 (January 3, 2003) "Borinic Acid Complex Antibiotics"
- 5) US Provisional Application No. 60/434,849 (January 3, 2003) "Borinic Acid Complex Antibiotics"
- 6) US Provisional Application No. 60/478,921 (June 16, 2003) "Borinic Acid Antibiotics Resistant to Hydrolysis"
- 7) US Provisional Application No. 60/486,005 (July 10, 2003) "N-Amino-Azole And Hydroxy-Azole-Borinate Complexes"
- 8) US Patent Application No. 09/578,991 (December 18, 2003) – "Antibiotics Containing Borinic Acid Complexes and Methods of Use"
- 9) PCT Application No. PCT/US00/14479 (December 18, 2003)- "Antibiotics Containing Borinic Acid Complexes and Methods of Use"
- 10) US Provisional Patent Application No. 60/579,421 (filed June 17, 2004) (pending)  
Title: "Boron-Containing Compounds and Methods of Use"
- 11) US Provisional Patent Application No. 60/579,476 (filed June 15, 2004) (pending)  
Title: "Anti-Parasitic Uses of Borinic Acid Complexes"

- 12) US Provisional Patent Application No. 60/579,419 (filed June 15, 2004) (pending)  
Title: "Anti-Viral Uses of Borinic Acid Complexes"
- 13) US CIP Patent Application No. TBD (filed June 14, 2004) (pending)  
Title: "Antibiotics Containing Borinic Acid Complexes and Methods of Use" (refilling of the Dec 18, 2003 application)
- 14) US Provisional Patent Application No 10/868,268.(filed June 15, 2004) (pending)  
Title: "Hydrolytically-Resistant Boron Therapeutics"
- 15) PCT Patent Application No. PCT/US04/18765 (filed June 17, 2004) (pending)  
Title: "Hydrolytically-Resistant Boron Therapeutics"

#### **(4) Scientific Progress and Accomplishments**

When work on this contract began, Anacor had two potential lead compounds, AN0002 and AN0004. The synthetic methods for AN0002 and AN0004 were optimized and scaled up from the gram level to the kilogram level. Over a kilogram of GLP-grade AN0002 and AN0004 were made, the analytical methods were validated, reference standards were developed, and no polymorphs were found. AN0002 and AN0004 were active against both gram positive and gram negative standard clinical pathogens and were bactericidal in their mode of action. They were most active against tularemia with MIC values as low as 0.01 µg/mL. The MIC versus anthrax was 6 µg/mL, with the MIC values against MSSA, MRSA, PSSP, and PRSP being 0.25 - 4 µg/mL for all. Time-kill curves for the lead compounds were generated against *Staphylococcus aureus* and *Streptococcus pneumoniae*, anthrax, and tularemia. Effective killing was seen at doses ranging from the MIC to 4x the MIC. MIC<sub>90</sub> values were obtained for these leads against *Staphylococcus aureus* and the results indicated that the MIC did not vary against sensitive or resistant bacterial strains. Frequency of resistance studies against *Streptococcus aureus* indicated the frequency of resistance for these leads were  $< 10^{-9}$ , which was the sensitivity limit of this assay. The protein binding for AN0002 and AN0004 was 60-70%, with results for mouse, rat and human plasma being similar. However, AN0002 and AN0004 both had a very short half life in the mouse and poor bioavailability based on LC/MS/MS analysis. The very rapid clearance of these compounds prevented achieving potentially efficacious plasma levels of the compounds after oral dosing. Because of this, their development as oral therapeutics was stopped and instead we evaluated the use of AN0002 as an intravenous therapeutic in either a rat or a mouse *Streptococcus pneumoniae* thigh model. A series of studies were conducted to make sure the intravenous infusion formulation was safe, to make sure AN0002 was safe by intravenous infusion, and to make sure the plasma levels achieved had the potential for efficacy based on our *in vitro* microbiology results. In order to achieve a plasma exposure of 5 µg/mL, we were forced to use infusion volumes of the organic formulation and compound that were borderline toxic. There did not seem to be any way to increase the dose any further, and it was clear from the time kill studies that higher exposures were likely needed for any serious chance at *in vivo* efficacy. Based on these findings, the development of AN0002 was halted.

During the entire effort to develop the early leads, medicinal chemistry and microbiology efforts were underway trying to understand what structural features were important for each aspect of the compound's performance. Through a rational medicinal chemistry approach, over 200 compounds were synthesized and tested. From this effort, compound AN0128 was discovered and emerged as a potential candidate drug in the early part of 2003. This compound has been proven to be much more chemically stable and less toxic to human cells than the early leads were, and has rodent PK profiles indicating the potential for once daily dosing in man (half life of 5-6 hours, bioavailability > 50% in both mice and rats). AN0128 has strong microbiological activity against gram positive bacteria, with an MIC of

1-2 µg/mL for the standard gram positive pathogens (MSSA, MRSA) and an MIC of 0.5 µg/mL against anthrax.

As was shown in Q1/Q2 2003, AN0128 proved to be essentially completely protein bound, leaving the free plasma levels insufficient to effectively kill the bacteria. Thus, when AN0128 was tested in the pulmonary anthrax model, it provided no protection against the ensuing lethality. As a result, Anacor shifted screening efforts to focus on potential effects of protein or serum on bacterial killing by routinely testing our compounds against *S. aureus* in the presence and absence of 100% fetal calf serum. We evaluated both historic compounds and all new compounds using this screen and found that borinic acid picolinate ester compounds, as a class, had extremely high protein binding. The borinic acid quinoline ester complexes, in contrast, still showed serum effects on bacterial killing, but these were much more moderate.

Anacor then diversified its search for active compounds by having medicinal chemistry create new AN0128 analogs with lower protein binding, while pharmacology mined the quinoline library for stable compounds with reasonable pharmacokinetics. After an extensive campaign, it became apparent that modification to any part of the picolinic acid compounds did not yield active compounds without the severe protein binding concerns. In contrast, we identified quinoline compounds that were indeed stable in plasma and that had reasonable mouse pharmacokinetics. It appeared that AN0002 and AN0004 were not actually typical representatives for their class. Based on these results, Anacor shifted focus back on the quinoline series and an intense screening effort was performed to derive structure activity relationships (SAR) for the quinoline series in Q3, 2003. Within a 2 month period we developed analytical methods for over 20 compounds, and then obtained their plasma stability and mouse pharmacokinetics. Anacor was able to identify specific traits that were detrimental to the molecule. For instance, having too many halogens on the phenyl rings caused toxicity to eukaryotic cells. Placing either methyl, methoxy or thiomethyl groups at the *para* position of the phenyl rings caused the compounds to be unstable. Having a fluorine at the *para* position of the phenyl ring induced greater serum effects on the MIC than if it was placed at the *meta* position.

With these SAR guidelines in hand, the screening success rate increased tremendously for creating compounds with proper microbiological efficacy, with minimized serum effects on bacterial killing, with minimal eukaryotic toxicity, and with plasma stability. The next major objective was to optimize the pharmacokinetic profile and this was the focus of Q4, 2003. We had compounds with a long half life, with very good oral bioavailability, with good volume of distribution ( $V_{ss}$ ) and with high maximum plasma concentrations ( $C_{MAX}$ ). The best leads, however, tend to have only 3 of these 4 traits. Two of the compounds, AN0185 and AN0189 have a very low  $V_{ss}$ , suggesting they remain primarily within the bloodstream and may not penetrate into infected tissues. Compound AN0185, however, achieved plasma exposures above 600 µg/mL when given at 300 mg/kg po to fasted mice. Compound AN1288 has a much better  $V_d$ , but its  $C_{MAX}$  was much lower than AN0185, with values of about 130 µg/mL when given at 100 mg/kg to fasted mice. And AN0189 also has a modest  $C_{MAX}$  of 82 µg/mL when given at 200 mg/kg po to fasted mice. We showed that if either AN0189 or AN1288 was administered at 300 mg/kg QID to mice, a 50% reduction in total thigh bacterial counts in neutropenic mice was achieved. The QID dosing was needed due to the modest  $C_{MAX}$  values, and the protection afforded was very small, but never-the-less real. This efficacy study did show that once we achieved the appropriate plasma exposure, our compounds could be effective in rodent efficacy models. Data from an anthrax septicemia study also showed a small protective effect of both AN0185 and AN1288 to delay the onset of death caused by an intravenous injection of vegetative anthrax. These studies demonstrated that our compounds could work in rodent efficacy models.

In Q4, 2003, we successfully optimized the quinoline compound structure to achieve very high plasma exposures. However, even using compounds with fully optimized pharmacokinetics we were never able to achieve robust *in vivo* efficacy. We found protein binding was more of a concern for bacterial infections than for other pathogens such as parasites, and in Q1, 2004 we were able to demonstrate robust efficacy in mouse parasitic disease models of malaria and African sleeping sickness. The success with parasites validated that the technology could work *in vivo*, and pointed towards protein binding being the most critical challenge to solve for the anthrax effort.

Through a concentrated effort in Q1, 2004, we discovered structural changes that reduced protein binding by 40-60%. We identified that for reduced protein binding we needed a basic nitrogen near the boron atom and located on one of the rings of the head group. This could be accomplished by using either a *meta*-pyridine or a *para*-pyridine. It can also be accomplished by adding a dimethylaminomethyl or a diethylaminomethyl group at either the *meta* or *para* position of the phenyl ring. You need only one of the basic nitrogen groups on one head unit and the other head unit can be either a substituted phenyl ring or a vinyl group.

These structural options allowed us to create parallel series of compounds to optimize for pharmacokinetics, safety, and efficacy. We found the dimethylaminomethyl series to tend towards having a lower maximum plasma level and a higher Vss than the pyridine series. This differentiation allowed us to test whether compounds with a high Vss would be more effective in the pulmonary anthrax rodent model and then focus on whichever structural series had the most promise.

In March, 2004, we tested AN1539 in our *S. aureus* mouse neutropenic thigh model. AN1539 is a meta-pyridyl complex with a 4-chlorophenyl as the second head group and an 8-hydroxyquinoline tail unit. Protein binding for AN1539 was 59%, it had a half life of > 5 hours and we approximated the Vss to be 400-600 mL/kg. This Vss suggests the drug will prefer to stay in the blood stream more than the tissues. The maximum plasma level after a 300 mg/kg oral dose was 131 µg/mL, but with the volume of distribution we would anticipate the thigh tissue levels of AN1539 to be less than the plasma values. Pilot safety studies at the 300 mg/kg dosing level showed the compound was well tolerated under these conditions. The MIC for AN1539 versus *S. aureus* was 2 µg/mL. We tested AN1539 in the *S. aureus* thigh model using two different dosing paradigms. The first paradigm was to give 300 mg/kg orally twice on the first day separated by 8 h. The second paradigm was to give 300 mg/kg orally for the first dose and then give 150 mg/kg orally every 6 h after that. The first dose of AN1539 was administered 3 h before the challenge with *S. aureus* and the mice were sacrificed 24 h after inoculation. The thigh tissues were harvested, processed and plated for bacterial load. AN1539 was more effective using the second dosing paradigm and decreased the thigh bacterial load by 93% (1-1.5 log drop). The first dosing paradigm also showed a small reduction in thigh bacterial load, but the extent of reduction was much less than that obtained using the second dosing paradigm.

At the March 26, 2004 meeting with DARPA and USAMRMC, Anacor received clearance to extensively use the University of New Mexico (UNM) anthrax testing facilities. During the next 4 months UNM evaluated compounds *in vitro* for their MIC against anthrax, and evaluated selected compounds in either their intravenous vegetative anthrax mouse model or their pulmonary anthrax mouse model.

In April we chose 4 compounds for evaluation in the pulmonary anthrax model. Three of these compounds (AN1558, AN1565, and AN1566) had low Vss (595, 292, and 651 mL/kg, respectively) and AN2301 had a high Vss (2391 mL/kg). Compounds AN1558 and AN1566 both had a long plasma half life (> 4 h), whereas AN1565 and AN2301 had moderate plasma half lives (2.1-2.4 h). AN2301 provided a short life extension for the infected mice (< 24 h), whereas the three compounds with a low Vss did not provide any protection.

We tested 8 compounds in the pulmonary anthrax mouse model in May. Of the 8 compounds tested, 3 showed enhanced survival of the mice. Compounds AN1568 and AN2453 provided significant life extension by about 1-1.5 days. Compound AN2478 gave a similar result, but because one of the control mice failed to die from the inoculation statistical significance was not reached. In contrast, compound AN2382 looked very promising from computations, but had a lower Vss and failed to give robust protection of the mice. These results strongly encouraged us to focus on compounds with high Vss.

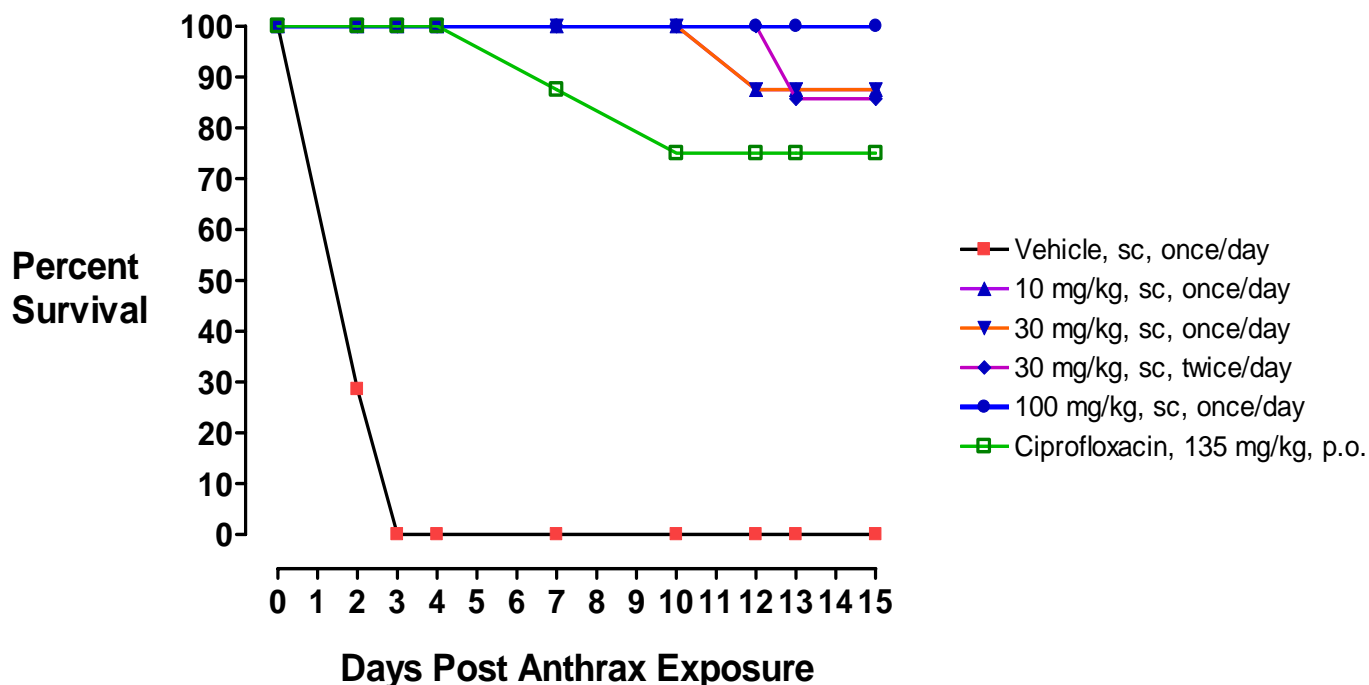
Exposure studies using CD1 mice evaluated the tissue levels of the compounds versus the plasma levels. We tested over a dozen compounds and found that with higher Vss values we did indeed find higher tissue levels as compared to plasma concentrations. At a Vss of about 800-1,000 mL/kg, the ratio is about equal between thigh tissue and plasma. For compounds with higher Vss values, the thigh levels were higher than plasma levels (e.g., Vss = 2,000 mL/kg, thigh levels were 2X plasma; for Vss = 3,000 mL/kg, thigh levels were 3X plasma) and when the Vss was below 1,000 mL/kg, then plasma levels

exceed tissue levels (e.g.,  $V_{ss} = 500$  mL/kg, thigh levels were 0.5X plasma levels). These findings allowed us to predict what tissue exposures would be for the compounds. For compounds with high  $V_{ss}$ , this computation appeared to be predictive of success in the mouse pulmonary anthrax model.

We tested 5 compounds in the pulmonary anthrax mouse model in June. Of these, compound AN2500 performed the best by preventing 20% of the mice from dying from anthrax. By the end of the study the AN2500-treated mice looked fine and healthy, but were clearly very sick on days 2-6. In an effort to try to get 100% survival using AN2500, we repeated this study using a higher, more intense dosing paradigm (300 mg/kg, po, on day 1 followed by 150 mg/kg, po, in the mornings and 300 mg/kg, po every evening for the remaining days). Unfortunately, none of the AN2500-treated mice survived. One explanation is that we pushed the AN2500 dose up to its toxic level in the infected mice, thereby mitigating any protection afforded by preventing anthrax toxicity. In July a third study was done using AN2500 at two different round-the-clock dosing paradigms to maximize exposure and minimize maximal plasma levels in the vegetative-anthrax, intravascular-inoculation mouse model. Results from this study did not show efficacy, and instead confirmed that AN2500 was toxic to the mice using the higher dosing paradigms. Thus, the maximal efficacy for AN2500 was 20% survival.

We also tested AN2453 and AN1568 for a second time, but with a slightly elevated dosing paradigm. These are the compounds that provided a 1-day life extension in June. We did not find any enhancement in activity of these compounds using the higher dosing strategy. In fact, these compounds did not yield a significant improvement versus vehicle in this study. However, this may have been due to the vehicle control mice surviving the anthrax challenge in this study better than in the first trial. Compounds AN1595 and AN1494 both failed to protect in this model. In July we screened 4 more borinic acid ester compounds (AN1610, AN2479, AN2596, AN2610) in the pulmonary anthrax mouse model. The best of these compounds, AN1610, extended lifespan by 1 day, but the remainder showed no effect.

We next screened AN0900, a semi-synthetic glycopeptide, in the vegetative-anthrax, intravascular-inoculation mouse model. AN0900 was administered 1 h after the anthrax inoculation as either a 25 or 50 mg/kg iv bolus. A second, final dose was also given on the morning of day 2. By 26 h after inoculation, all vehicle control mice had died, but by day 7 after inoculation, all mice treated with either dose of AN0900 were all alive and healthy. None of the AN0900 anthrax-treated mice showed any signs of anthrax toxicity during the study, which is very analogous to the positive control drug Ciprofloxacin. In September, 2004 we evaluated AN0900 in the UNM pulmonary anthrax model. AN0900 was given as a once daily subcutaneous (sc) dose of either 10, 30 or 100 mg/kg, or as a twice daily sc dose of 30 mg/kg. Ciprofloxacin was given as a once-daily oral dose of 135 mg/kg. Dosing with both AN0900 and ciprofloxacin was initiated 1 h after inoculation with  $1.66 \times 10^4$  spores, and continued through day 4 of the study. As shown in the following figure AN0900 provided long-lasting protection at all doses tested and performed better than ciprofloxacin under these conditions.



Currently, ciprofloxacin is the drug of choice for anthrax clinically and out performs other existing antibiotics in the preclinical anthrax models. Unfortunately, resistance against ciprofloxacin has already emerged and it would not be difficult for bioterrorists to engineer an anthrax strain against which ciprofloxacin will be useless. AN0900 may be one of the first new compounds with a unique mechanism of action that may prove as efficacious as ciprofloxacin.

Even though DOD financial support has stopped for this program, the robust efficacy shown by AN0900 in the DOD-standard mouse anthrax models warrants further drug development for this compound. Anacor is currently considering options for how to proceed with this important effort.

## (5) Technology Transfer

None.